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Production, purification and characterization of lipase enzyme from *Bacillus* species

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ABSTRACT:
Extracellular lipase was isolated and purified from culture broth of *bacillus* species. The optimum pH and temperature for activity of lipase was found to be 8.0 and 40ºC. The lipase was found to be stable in the pH range 6.5–8.5 and temperature range 30–60ºC. The enzyme was incubated with 50% organic solvents showed a good stability and enzyme with 30% and 70% showed slight stability decreases. Fe^{2+}, Cu^{2+}, at 10mM enhanced hydrolytic activity of the lipase. Interestingly, K^{+} ions resulted in a maximal increase in lipase activity but Na^{+}, Mg^{2+}, and Ca^{2+} ions showed an antagonistic effect on this enzyme. EDTA at 10mM concentration inhibited the activity of lipase but Tween80 and Triton X-100 strongly stimulated lipase activity Sodium perchlorate and SDS (10mM) decreased 98% of original activity of lipase. The lipase enzyme activity was investigated using various concentration of *p*-nitrophenyl palmitate (pNPP). The Vmax and Km was calculated by various kinetic plots.
Keywords: Hydrolytic activity, organic solvents, antagonistic effect, sodium perchlorate, *p*-nitrophenyl palmitate, kinetic plots.

INTRODUCTION

The major share of the industrial enzyme market is occupied by hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. In recent times, lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) have emerged as key enzymes in swiftly growing biotechnology, owing to their multifaceted properties, which find usage in a wide array of industrial applications, such as food technology, detergent, chemical industry and biomedical sciences [1-3]. Lipases are ubiquitous in nature and are produced by various plants, animals and microorganisms. Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry. The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier. Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains [4]. Of these, the important ones are: *Achromobacter* sp, *Alcaligenes* sp., *Arthrobacter* sp., *Bacillus* sp., *Burkholderia* sp., *Chromobacterium* sp and *Pseudomonas sp*. Of these, the lipases from *Bacillus* bacteria are widely used for a variety of biotechnological applications [5,6]. Use of lipases for synthesis has numerous advantages over chemical synthesis. This approach requires lipase preparations to be stable in the organic solvents and other salts. Generally the enzymes are inactivated or give very low rate of reactions in non-aqueous media; therefore, search for solvent stable enzymes has been an extensive area of research [7,8].Most of the microbial lipases are active at alkaline pH (pH 7.0–9.0) with a few exceptions [9]. Thus the activities of lipases are highly pH dependent and any alteration in pH of the reaction mixture is likely to affect the catalytic potential of the lipases. The metal-ions are also effective antagonists and modulators of lipases. Chelating agents like EDTA inhibit the activity of metallolipases and not the other ones [10, 11]. Structural elucidation of lipases has shown that catalytic site of most lipases resembles that of serine proteases [12].Present work describes the distinct features of this lipase viz nature of lipase and stability in surfactants, solvents, salts.

MATERIAL AND METHODS

Chemical and reagents

p-nitrophenyl palmitate was purchased from sigma-Aldrich,India. The medium components were procured from Himedia Ltd, Mumbai, India. All other solvent and chemicals used during the experiments were of analytical grade.

Culture condition

The bacterial strain grown in the NB medium (2 g/l yeast extract, 1 g/l beef extract, 5 g/l peptone and 5 g/l NaCl, pH 7.2) for 24 h at 30°C was used as the inoculum.

Production of Lipase

The lipase producting *bacillus* species was inoculated in 500 ml Erlenmeyer flask with 250 ml of the production medium. The production medium contained % (w/v): glucose 0.5, ammonium sulfate 0.4, peptone 0.5, magnesium sulfate 0.01, potassium dihydrogen phosphate 0.1, sodium chloride 0.2 and olive oil 1.5 (%v/v). The medium pH was adjusted to 7.5 with 0.1 M NaOH. The production was seeded with 5% of inoculums. The fermentation was carried out at 120 rpm in an incubator shaker maintained at 40°C for 12 hr. After 12 hr the culture medium was centrifuged at 10,000rpm in a cooling centrifuge at 4°C for 15 minutes.

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And the supernatant was collected for purification process.

Enzyme assay method

Lipase activity was assayed by the spectrometric method by measuring the micromoles of *p*-nitrophenol released from *p*NPP. In brief a stock solution of 50mM of *p*NPP was prepared by dissolving in acetonitrile and butanol in 1:1 ratio. The reaction mixture contained 75µl of *p*NPP stock solution, 25µl enzyme, and Tris buffer (0.1M, pH 8.5) to make a final volume of 3ml. The reaction mixture was incubated at 55ºC for 10 min in water bath. Chilling under tap running water was done to stop the reaction. The absorbance of *p*-nitrophenol released was measured at 410 nm using UV spectrophotometer. One unit of lipase activity was defined as micromoles of *p*-nitrophenol released from *p*NPP per ml per min under standard assay condition.

Crude Enzyme purification

The cell-free supernatant was prepared from the bacterial by centrifugation (10,000 rpm, 15min) of culture broth. The culture supernatant was concentrated by ultrafilteration with an amicon stirred cell with a PM-10 membrane, until a 20-fold concentration was achieved. The enzyme precipitated with help of ammonium sulfate. The ammonium sulfate was added in concentrated supernatant up to final saturation 50% with slow mixing and left at 4ºC for 6 hr. After 6 h solution mixture was centrifugation at 10,000 rpm for 30 min. The precipitate was then discarded. Then ammonium sulfate was added to the supernatant up to final concentration of 60% (w/v). After standing in the ammonium sulfate solution for 12 h at 4°C, the precipitate was collected by centrifugation at 10,000 rpm for 30 min and dissolved in 20mM Tris-HCl buffer. The resuspended crude enzyme solution was dialysis against 20mM Tris-HCl buffer at 4 °C for 12 h to remove the residual ammonium sulfate [13].

Effect of pH and temperature

To evaluate the effect of reaction temperature and pH on lipase activity, the lipase activity was assayed at various temperatures (30, 40, 50, and 60 °C) and pH (6.5, 7, 7.5, 8, and 8.5) values. The thermostability determination the purified lipase was incubated at different temperature ranging from 30 to 60°C for 1 hr in 0.1M Tris-HCl buffer. To measure the pH stability of lipase enzyme was incubated at 35°C for 1 hr in various pH of 0.1M Tris–HCl buffer. The residual lipase activity was assayed under standard assay conditions.

Effect of organic solvent

The effect of different organic solvents on lipase activity was analyzed by incubating enzyme mixture with organic solvents (30, 50, and 70%) for 1hr at 30ºC. For control distilled water was added instead of organic solvents. Lipase activity was assay using pNNP as substrate.

Effect of various compounds on purified lipase

Enzymes were preincubated for 1 h at 30ºC (pH 7.0) and in 0.1M Tris-HCl buffer with various ions (one at a time).The ions are $MgCl_2$ (10mM), $CuSO_4$ (10mM), $CaCl_2$ (10mM), NaCl (10mM), $FeSO_4$ (10mM).The effect of various detergents and surfactants was separately added to the reaction mixture at a final concentration of 1.0mM. After 1hr incubation at 30°C, the lipase activity was assayed.

Determination of Km and Vmax

Hyperbolic Regression Software 3.95 was used for determining the Km and Vmax from various kinetic plots. Km and Vmax constants were determined by measurement of enzyme activity with various concentrations of *p*NPP substrate. The apparent kinetic parameters were estimated from kinetic plots and Km and Vmax were calculated for each experiment by liner and non-linear regression analysis [14,15].

RESULTS AND DISCUSSION

Effect of temperature on Stability

The effect of temperature on lipase activity and stability was measured using *p*NPP as a substrate. To test the effect of temperature on lipase activity, assays were performed for 1 h at various temperatures. The lipase was most active in the temperature range 30–60°C, with maximal activity at 40°C (Fig.1).

Effect of pH on Stability

The effect of pH on lipase activity at 35°C with *p*NPP as substrate was examined at various pH values. The enzyme was active in the pH range 6.5–8.5 and the optimal pH was shown to be 8 in 0.1M Tris–HCl buffer (Fig.2).

Stability in organic solvent

Fig.3 shows the effect of organic solvent on crude enzyme. It was observed that enzyme incubated with 30% methanol showed a good stability and enzyme with 50% methanol showed comparatively better stability and found to show the maximum stability of enzyme. But increase in concentration of methanol the stability decreases. For stability check with acetone, 50% of solvent shows better stability and increase or decrease in solvent concentration reduces the enzyme stability. For acetontrile solvent the stability was reduced with increase or decrease in concentration compared the stability found at 50% concentration.

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Effect of Metal ions on lipase activity

The effect of various metal ions on crude enzyme and its activity was studied. From fig.4 shown that the presence of Mg^{2+}, Cu^{2+}, and K^{+} shows better activity of enzyme compared to Na^{+}, Fe^{2+}, and Ca^{2+} . The effect of metal ions on enzyme was checked by incubating and the resulting lipase activity was shown in fig.4.

Effect of Surfactants and chelating agent on lipase activity

From the fig.5 Triton X 100 and Tween 80 presence with the enzyme show enhancement in its enzyme activity and the ionic surfactants observed to show inhibition of the enzyme activity. SDS shows the maximum inhibition of the enzyme and Triton X 100 shows maximum enhancement of the enzyme.

Characterization of *Bacillus* lipase

The kinetic parameters for lipase were determined by Michaelis Menten Hyperbola (Fig.6). It showed Km of 86.6 (mg/ml) and Vmax of 15.57 (IU/ml) towards pNPP as substrate. From Lineweaver–Burk plot (Fig.7) given Km of 171.9(mg/ml) and Vmax of 22.45(IU/ml).The constant Vmax 16.33 (IU/ml) and Km 99.6 (mg/ml) was determine using Hanes plot (Fig.8). The Eadie Hofstee plot given that Vmax 16.75(IU/ml) and Km 104.4 (mg/ml) (Fig.9).

CONCLUSIONS

The optimum pH and temperature for activity of lipase were found to be 8.0 and 40ºC. The effect of organic solvent on crude enzyme showed a good stability at 50% solvent and increase or decrease in solvent concentration reduces the enzyme stability. The effect of metal ions on lipase activity was stimulated by Fe^{2+}, Cu^{2+} and K^{+} and Cu^{2+} and inhibited by Na^{+}, Mg^{2+}, and Ca^{2+}. Lipase activity was strongly increased by Tween 80 and Triton X-100 but inhibited by EDTA and sodium perchlorate and SDS. Further kinetics studies are cared out by hydrolysis of *p*NPP substrate by lipase enzyme.

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Table 1: Vmax and Km for various kinetics plots on lipase activities

S.No	Kinetic Plot	Vmax	Km
1	Michaelis Menten Hyperbola	15.57	86.6
2	Lineweaver-Burk plot	22.45	171.9
3	Hanes plot	16.33	99.6
4	Eadie Hofstee	16.75	104.4

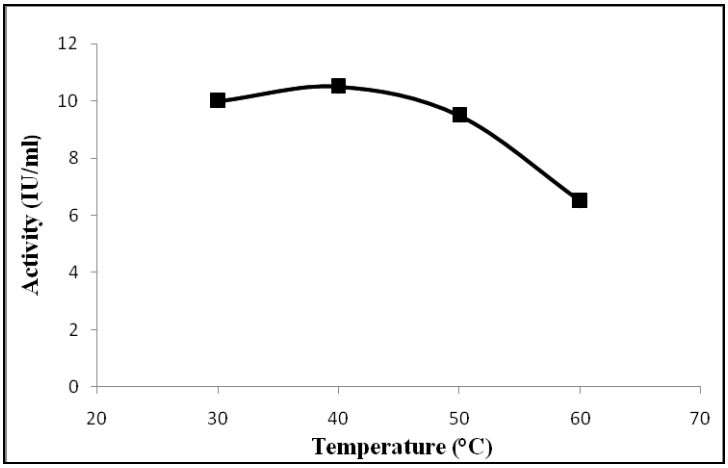

Fig.1 Effect of temperature on lipase activity

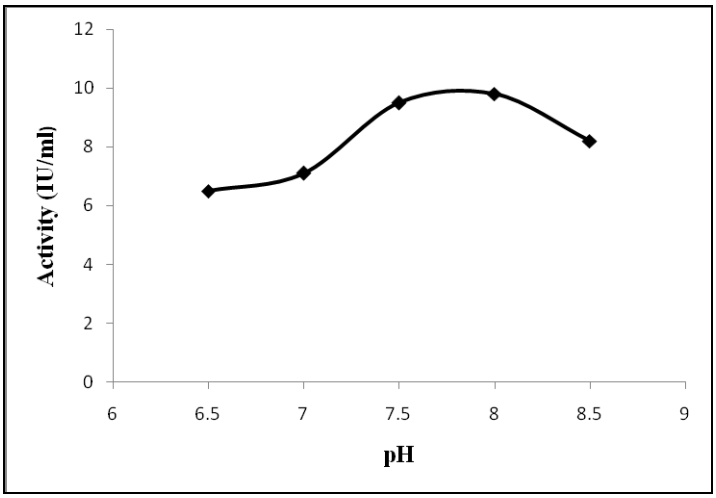

Fig. 2 Effect of pH on lipase activity

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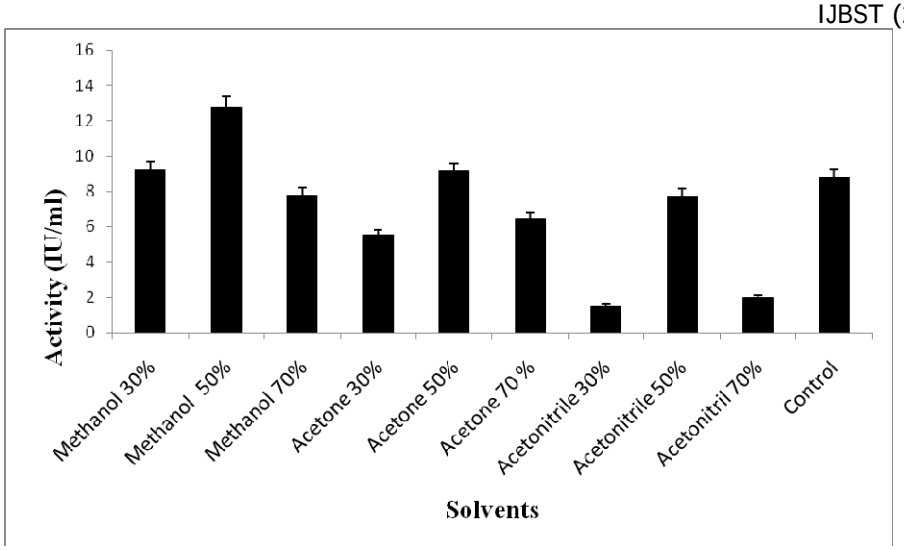

Fig 3. Effect of organic solvent on lipase activity

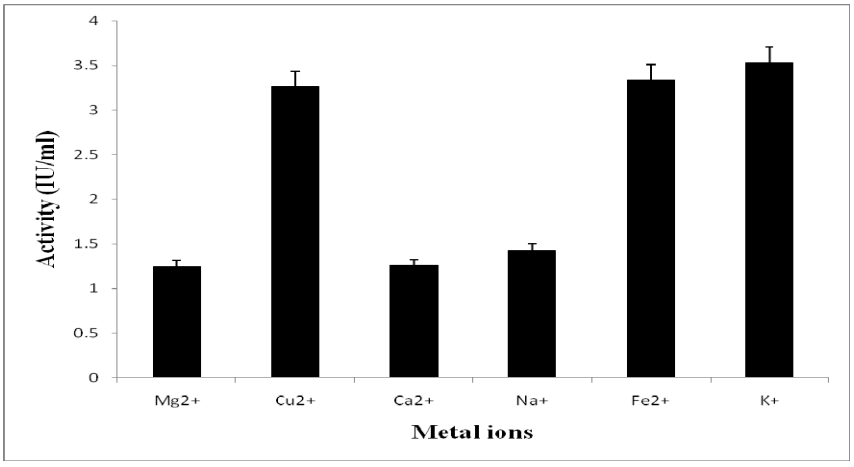

Fig. 4 Effect of metal ions on lipase activity

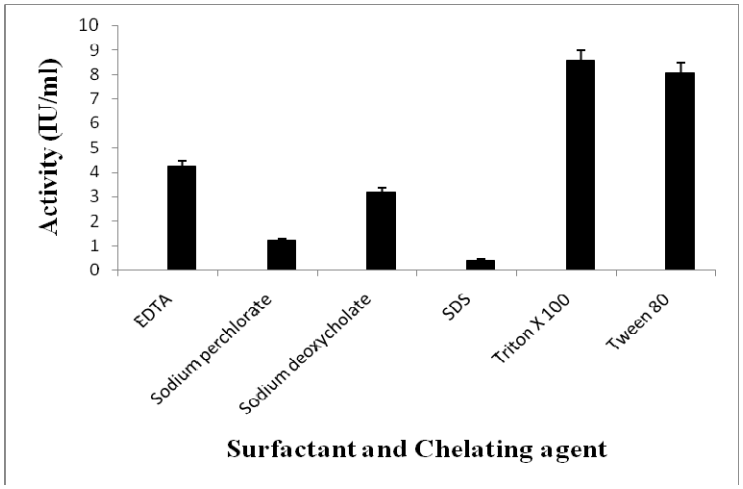

Fig. 5 Effect of surfactants and chelating agent on lipase activity

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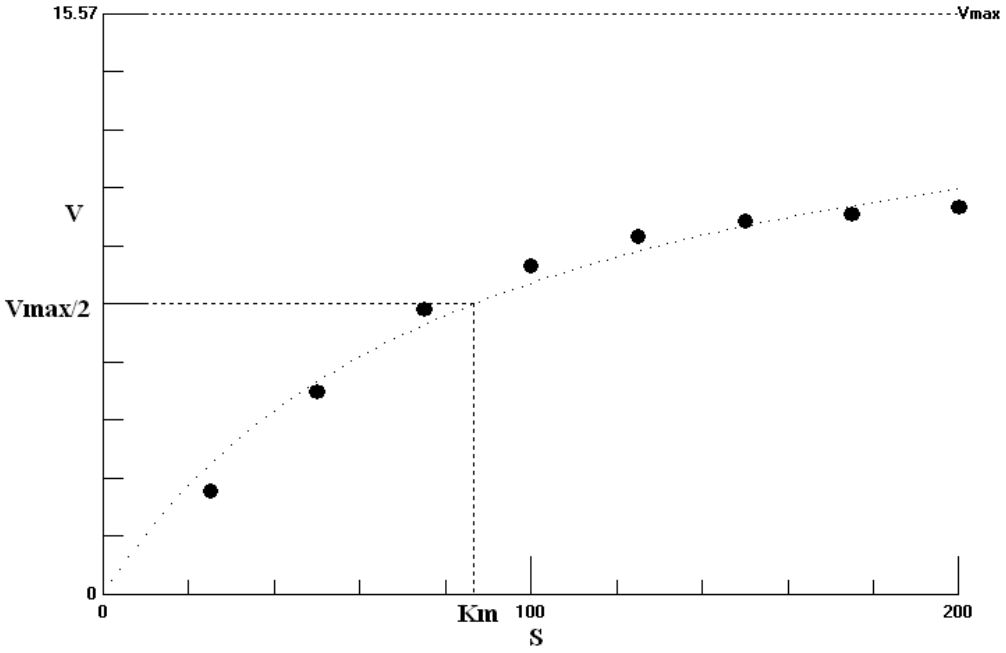

Fig. 6 Michaelis Menten Hyperbola for lipase activity

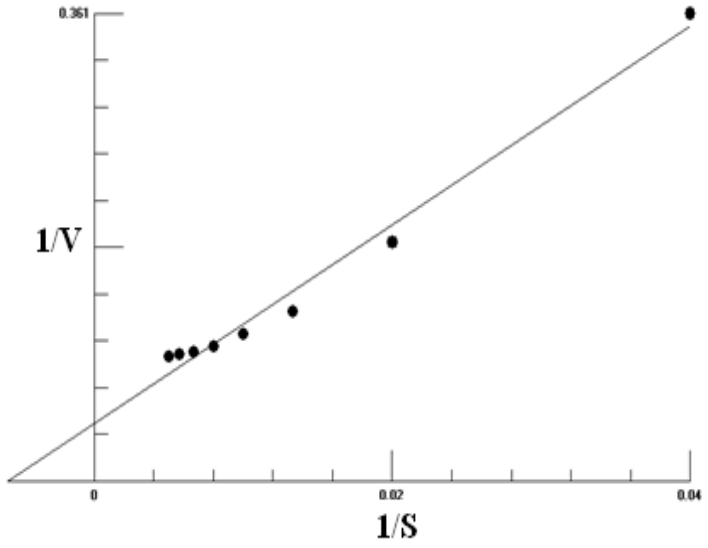

Fig. 7 Lineweaver-Burk plot for lipase activity

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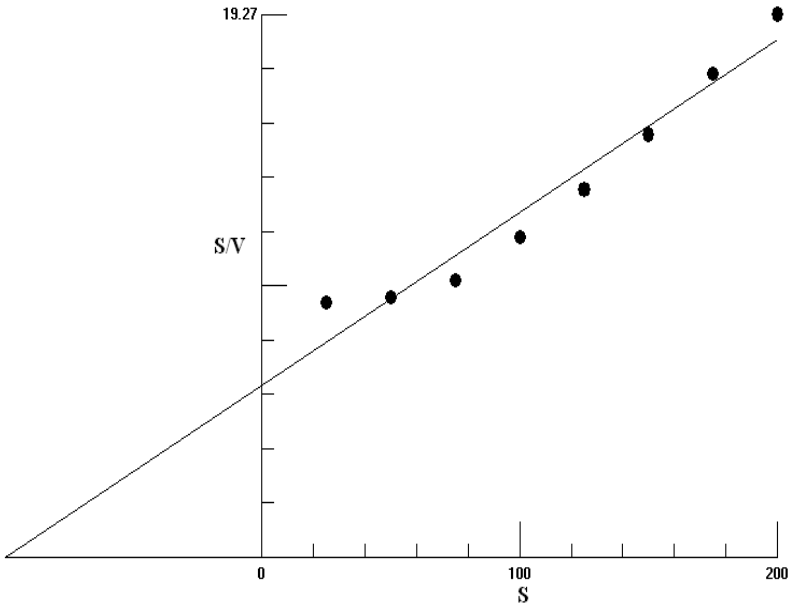

Fig. 8 Hanes plot of for lipase activity

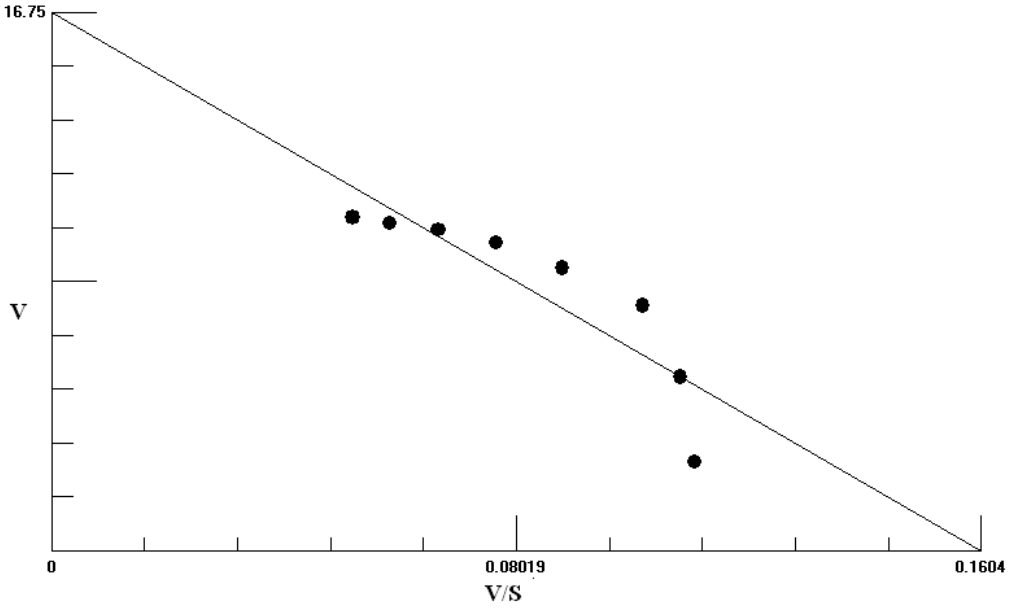

Fig. 9 Eadie Hofstee plot for lipase activity